USA300 and USA500 Clonal Lineages of Staphylococcus aureus Do Not Produce a Capsular Polysaccharide Due to Conserved Mutations in the cap5 Locus

RESEARCH ARTICLE

USA300 and USA500 Clonal Lineages of *Staphylococcus aureus* Do Not Produce a Capsular Polysaccharide Due to Conserved Mutations in the *cap5* Locus

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ABSTRACT **The surface capsular polysaccharide (CP) is a virulence factor that has been used as an antigen in several successful vaccines against bacterial pathogens. A vaccine has not yet been licensed against *Staphylococcus aureus*, although two multicomponent vaccines that contain CP antigens are in clinical trials. In this study, we evaluated CP production in USA300 methicillin-resistant *S. aureus* (MRSA) isolates that have become the predominant community-associated MRSA clones in the United States. We found that all 167 USA300 MRSA and 50 USA300 methicillin-susceptible *S. aureus* (MSSA) isolates were CP negative (CP$^-$). Moreover, all 16 USA500 isolates, which have been postulated to be the progenitor lineage of USA300, were also CP$^-$. Whole-genome sequence analysis of 146 CP$^-$ USA300 MRSA isolates revealed they all carry a *cap5* locus with 4 conserved mutations compared with strain Newman. Genetic complementation experiments revealed that three of these mutations (in the *cap5* promoter, *cap5D* nucleotide 994, and *cap5E* nucleotide 223) ablated CP production in USA300 and that Cap5E75 Asp, located in the coenzyme-binding domain, is essential for capsule production. All but three USA300 MSSA isolates had the same four *cap5* mutations found in USA300 MRSA isolates. Most isolates with a USA500 pulsotype carried three of these four USA300-specific mutations, suggesting the fourth mutation occurred in the USA300 lineage. Phylogenetic analysis of the *cap* loci of our USA300 isolates as well as publicly available genomes from 41 other sequence types revealed that the USA300-specific *cap5* mutations arose sequentially in *S. aureus* in a common ancestor of USA300 and USA500 isolates.**

IMPORTANCE **The USA300 MRSA clone emerged as a community-associated pathogen in the United States nearly 20 years ago. Since then, it has rapidly disseminated and now causes health care-associated infections. This study shows that the CP-negative (CP$^-$) phenotype has persisted among USA300 isolates and is a universal and characteristic trait of this highly successful MRSA lineage. It is important to note that a vaccine consisting solely of CP antigens would not likely demonstrate high efficacy in the U.S. population, where about half of MRSA isolates comprise USA300. Moreover, conversion of a USA300 strain to a CP-positive (CP$^+$) phenotype is unlikely *in vivo* or *in vitro* since it would require the reversion of 3 mutations. We have also established that USA300 MSSA isolates and USA500 isolates are CP$^-$ and provide new insight into the evolution of the USA300 and USA500 lineages.**

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Staphylococcus aureus is an important pathogen that causes a wide range of infections in health care and community settings. Methicillin-resistant *S. aureus* (MRSA) isolates in particular, which have become increasingly prevalent in the last decade, are resistant to nearly all β-lactams and are often multiply resistant to several classes of antibiotics. A vaccine that could protect against *S. aureus* infection would be important for public health, although development of an effective vaccine has remained elusive (1, 2).

Capsular polysaccharides (CPs) envelope the surface of many bacterial pathogens and have been the primary or sole protective antigen used in vaccines that are effective against certain serotypes of *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitidis* (serotypes A, CW, and Y) (3). Among encapsu-

lated *S. aureus* isolates, serotypes 5 and 8 prevail (4–9). Capsular polysaccharides 5 (CP5) and 8 (CP8) have similar trisaccharide repeating units but differ in the linkages between the sugars and the sites of O-acetylation of the monosaccharide residues (10). Previous reports have indicated that ~20% of *S. aureus* isolates fail to produce CP5 or CP8 (4–9).

The *S. aureus* CP protects the bacterium from host phagocytes (2); however, this protection can be overcome by CP-specific antibodies that enhance opsonophagocytic killing by human neutrophils (10, 11). Vaccines that contained CP5 and CP8 antigens conjugated to *Pseudomonas aeruginosa* exotoxoid A were tested for efficacy in patients with end-stage renal disease. In phase III clinical trials, these CP-based vaccines failed to protect against *S. aureus* bacteremia (12–14).

In the United States, the community-associated (CA)-MRSA clonal type USA300 has been the most frequently isolated *S. aureus* genotype from all body sites, including blood, surpassing the USA100 health care-associated MRSA strain type as a cause of nosocomial infection in some locales (15). USA300 MRSA strains are most often associated with CA skin and soft tissue infection (SSTI) (16) and belong to multilocus sequence type 8 (ST8). USA300 MRSA isolates characteristically contain the SCC*mec* type IV element (17), a phage carrying the genes encoding Panton-Valentine leukocidin (PVL) (18) and the arginine catabolic mobile genetic element (ACME) carrying the arginine deiminase cluster and the gene encoding the spermidine resistance factor, SpeG, which promotes skin colonization (19–21). Although they usually lack SCC*mec* IV, ACME, and PVL, USA500 MRSA isolates are the closest relative of USA300 among the members of the ST8 lineage and have been postulated to be the progenitor of USA300 (21).

We previously demonstrated that neither CP5 nor CP8 was produced by several USA300 MRSA clinical isolates obtained in 2004 and 2005 from our center in Chicago, IL (22). A subsequent study of isolates obtained during the same time period (2004 to 2005) from Washington, DC, also reported CP-negative (CP^-) USA300 MRSA strains (23). These studies suggested that the failure to produce a CP was a common trait among USA300 MRSA isolates circulating in 2004 through 2005, but this was not investigated subsequently.

A decade has passed since the CP^- USA300 MRSA isolates were described. Since then, USA300 MRSA strains have widely disseminated and increased in prevalence in the United States (15). It is not known if there are CP5-positive ($CP5^+$) USA300 variants in circulation or if the CP^- USA300 MRSA isolates identified in 2004 have become the sole or dominant USA300 clone.

Because CP5 and CP8 are components in two *S. aureus* vaccines under development (1, 24, 25), the aim of this study was to determine if currently circulating USA300 MRSA isolates produce a CP. Further, we evaluated whether the CP^- phenotype was shared by USA300 methicillin-susceptible *S. aureus* (MSSA) isolates and the closely related USA500 lineage (21, 26). Since we found that all USA300 and USA500 isolates were uniformly CP^-, we analyzed the evolutionary relationship between the *cap5* mutations in USA300 and USA500 isolates.

(This work was presented in part at the International Symposium on Staphylococci and Staphylococcal Infections, Chicago, IL, 2014.)

TABLE 1 Demographics of USA300 and USA500 clinical isolates

Parameter	No. of isolates					
	USA300			USA500		
	MRSA	MSSA	Total	MRSA	MSSA	Total
Colonization vs infection						
Colonization	134	20	154	0	0	0
Infection	33	30	63	14	2	16
Total	167	50	217	14	2	16
Geographic source						
Chicago	83	45	128	12	0	12
Los Angeles	84	3	87	1	0	1
San Francisco	0	2	2	0	2	2
Connecticut	0	0	0	1[a]	0	1
Total	167	50	217	14	2	16
Yr of isolation						
1995	1	0	1	0	0	0
1996	0	0	0	5	0	5
1997	0	0	0	2	0	2
2004	1	0	1	0	0	0
2008	37	0	37	5	0	5
2009	92	17	109	0	2	2
2010	33	21	54	0	0	0
2011	3	12	15	0	0	0
Unknown	0	0	0	2	0	1
Total	167	50	217	14	2	16

[a] This isolate is the USA500 reference strain NRS385.

RESULTS

CP serotyping. We chose for CP serotyping 233 clinical *S. aureus* isolates from our collection that were identified as either USA300 MRSA, USA300 MSSA, or USA500. Isolates were obtained from either colonized or infected body sites from subjects in Chicago, IL, Los Angeles, CA, San Francisco, CA, or Connecticut between 1995 and 2011, as described in Table 1. Clonal types consisted of (i) 167 USA300 MRSA isolates, (ii) 50 USA300 MSSA isolates, (iii) 14 USA500 MRSA isolates, and (iv) 2 USA500 MSSA isolates. The USA300 sample included 16 ACME *arcA*-negative USA300 MRSA isolates and 38 ACME *arcA*-negative USA300 MSSA isolates (as detailed in Table S1 in the supplemental material), since the absence of ACME *arcA* is atypical compared with other USA300 strains, and these isolates might exhibit altered CP phenotypes. Table S1 provides the typing characteristics of USA300 MSSA isolates, USA500 isolates, and ACME *arcA*-negative USA300 isolates.

USA300 MRSA and MSSA isolates. Capsule production was evaluated in 167 USA300 MRSA isolates. Although all USA300 MRSA strains tested carried a *cap5* locus, all were nonreactive to CP5 polyclonal antiserum, whereas the $CP5^+$ control strains Reynolds and Newman produced a signal (Fig. 1). Figure 1 also shows the nonreactivity to CP5 antiserum of the $CP8^+$ strains, ST80 and MN8, as well as the CP^- strains NCTC 8325-4 (27) and USA300 strain LAC. All 9 MRSA isolates that had a pulsotype consistent with USA300 but lacked ACME *arcA* were also CP^-. USA300 MRSA isolates were nonreactive with CP8 polyclonal antiserum, as expected, since they do not encode a *cap8* locus (data not shown). All 50 USA300 MSSA isolates tested were CP^-.

To address the possibility that USA300 strains might produce CP5 *in vivo* but not *in vitro*, we performed experiments similar to those described previously (28, 29). Both strain Newman and the USA300 MRSA strain CDC3 reacted with antibodies raised to

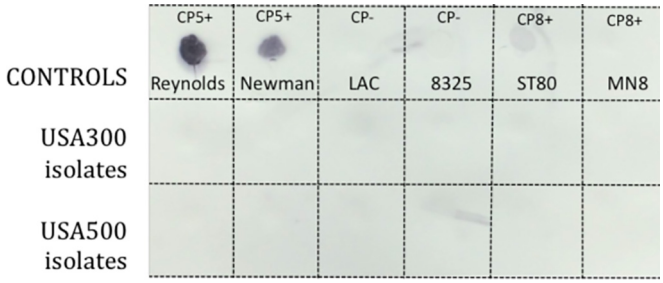


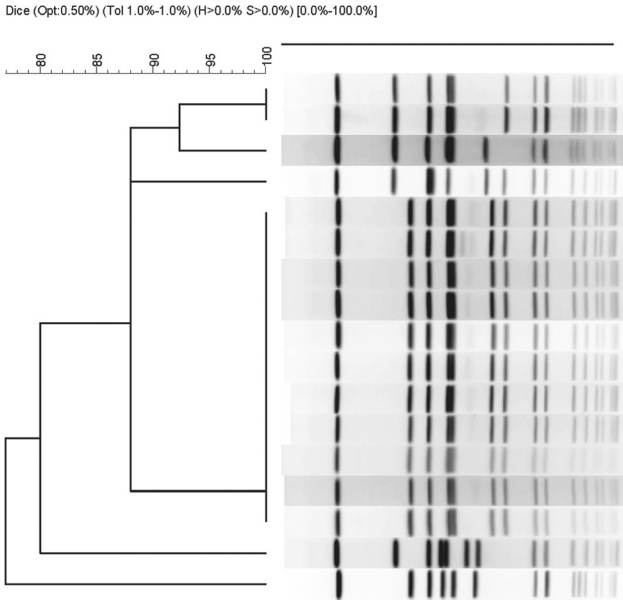
FIG 1 CP immunoblots of USA300 and USA500 clinical isolates. Capsule serotyping was performed by a colony immunoblot method with the use of CP5-specific antibodies as described previously (43). The first row shows the results from the CP5-positive ($CP5^+$) control strains Reynolds and Newman, the CP5- and CP8-negative (CP^-) control strains LAC and 8325, and the $CP8^+$ control strains ST80 and MN8. The second and third rows include six representative USA300 and USA500 clinical isolates, respectively.

killed acapsular *S. aureus* cells (see Fig. S1 in the supplemental material). The control $CP5^+$ strain Newman produced CP5 as visualized by the blue-staining Alexa Fluor 660-tagged secondary antibodies reacting with CP5 antibodies. In contrast, neither the inoculum of USA300 MRSA strain CDC3 nor the CDC3 bacteria isolated from mouse blood 6 h after bacterial challenge showed detectable reactivity with the CP5 antibodies. Similar results were obtained with samples of blood from mice challenged with USA300 strain LAC. Thus, our results show no evidence that USA300 MRSA isolates produce CP5 *in vivo*.

USA500 isolates. Due to the close phylogenetic relationship between USA500 and USA300 isolates (21, 30), we hypothesized that USA500 isolates might also be $CP5^-$. Figure 2 documents the pulsed-field gel electrophoresis (PFGE) patterns and relevant typing characteristics of the 16 USA500 isolates we tested for CP production. The USA500 isolates belonged to ST8, carried SCC*mec* type IV, and lacked ACME *arcA* (26, 30, 31) (see Table S1 in the supplemental material). However, most of these USA500 isolates carried *pvl* genes, which is atypical for USA500. The USA500 isolates belonged to three *spa* types: t064 (11 of 16 isolates), t008 ($n = 3$), or t211 ($n = 2$) (see Table S1). All 16 USA500 isolates were CP^- as represented in a panel in Fig. 1.

DNA sequence analysis of *cap* loci of USA300 MRSA isolates. To evaluate the genetic basis for the $CP5^-$ phenotype, we evaluated the *cap* loci from assembled whole-genome shotgun data from 146 CP^- USA300 MRSA isolates. Each strain carried an intact *cap5* locus but had 4 identical point mutations relative to the $CP5^+$ reference strain Newman (Fig. 3A).

The *cap5* promoter had a T→C replacement 73 bp upstream (−73) from the ATG translation start codon of *cap5A* (P_{cap5} −73). This was located in the *cap5* promoter in an inverted repeat (Fig. 3) known to be essential for transcription of *capABCDEF* (27, 32). We also identified a frameshift mutation within a polyadenine (AA) tract that begins at nucleotide (nt) 1006 of *cap5D* in strain Newman. This corresponds to nt 994 in *cap5D* of the USA300 reference strain because the annotations for the start codon of strain Newman and USA300 differ by four codons. This segment contains six A's in the wild-type (wt) *cap5D* gene (strain Newman) and seven A's in USA300 MRSA isolates, resulting in premature termination during translation (after 338 residues of the full-length Newman protein and 334 residues in USA300). There was also a G→T mutation at position 223 of *cap5E* (*cap5E*-223), which converts Asp to Tyr in the encoded protein at codon 75 (Asp75Tyr) and a T→C mutation in *cap5G*-478, resulting in a Phe160Leu conversion in the encoded protein. Additional nonconserved mutations were found in various *cap5* genes among USA300 isolates (data not shown) (33). The mutations in USA300 at *cap5E*-223 and *cap5G*-478 have not been previously recognized in USA300 isolates, whereas the mutations in P_{cap5} and *cap5D*



Strain name	*mec*A	Date of Isolation	*spa* Type	Cp5/8	CapE 223	CapG 478
22653	-	2009	t008	Neg	G	T
22654	-	2009	t008	Neg	G	T
12560	+	2008	t064	Neg	G	T
USA500ref	+		t064	Neg	G	T
021397A	+	1997	t064	Neg	G	C
021397C	+	1997	t064	Neg	G	C
042596A	+	1996	t064	Neg	G	C
050296C	+	1996	t064	Neg	G	C
052196C	+	1996	t064	Neg	G	C
073196B	+	1996	t064	Neg	G	C
090596B	+	1996	t064	Neg	G	C
092496B	+	1996	t064	Neg	G	C
11342	+	2008	t211	Neg	G	C
12021	+	2008	t211	Neg	G	C
12111	+	2008	t008	Neg	G	C
10833	+	2008	t064	Neg	G	T
USA300ref			t008	Neg	T	C

FIG 2 Pulsed-field gel electrophoresis (PFGE) documenting the SmaI digestion patterns of USA500 isolates relative to the USA500 reference strain NRS385 (USA500ref) and relevant typing characteristics. The USA300 reference strain (USA300ref) has PFGE pattern USA300-0114 and was obtained from the Network for Antimicrobial Resistance in *Staphylococcus aureus* (NARSA). "Cp5/8" refers to capsule polysaccharide types 5 and 8, "CapE 223" refers to the nucleotide at position 223 in *cap5E*, and "CapG 478" refers to the nucleotide at position 478 in *cap5G*.

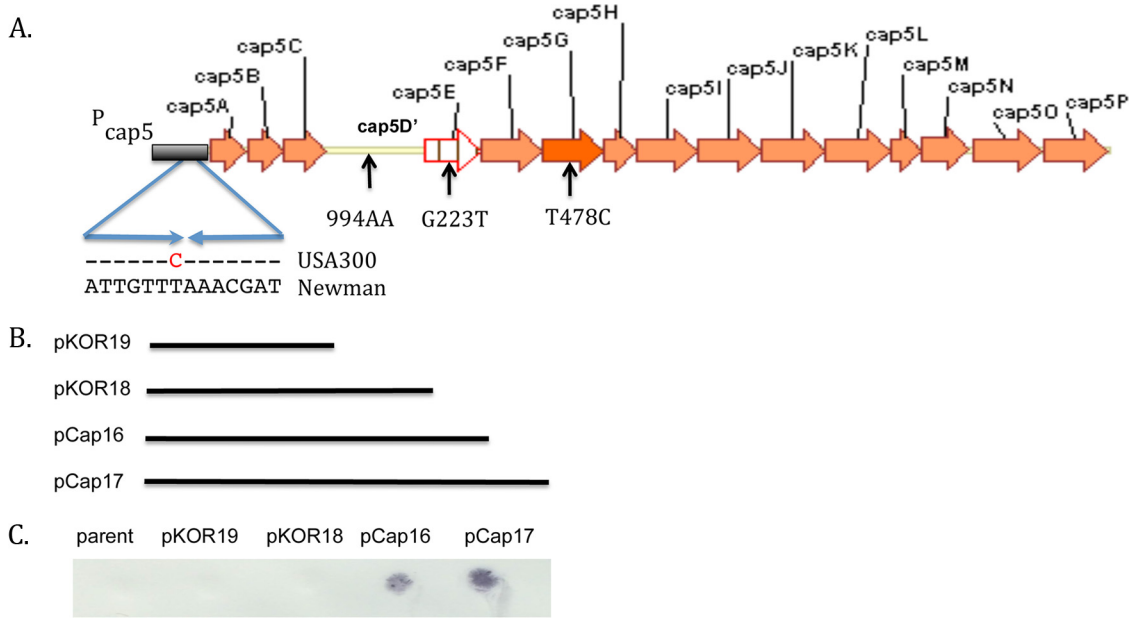


FIG 3 USA300 *cap5* locus showing universal mutations in USA300 relative to the *cap5* reference strain Newman. Orange arrows depict open reading frames (ORFs). "P_{cap5}" refers to the *cap5* promoter region. The inverted repeat sequence, as reported by Ouyang et al. (32), is shown as opposing arrows beneath P_{cap5} indicating the T→C mutation in strain USA300 relative to strain Newman. The yellow line indicates a disruption of the *cap5D* ORF due to insertion of an A at nt 994 in USA300 (994AA). The *cap5E* G→T mutation at nt 223 (G223T) and the *cap5G* T→C mutation at nt 478 (T478C) are indicated beneath each ORF. (B) Lines beneath the map indicate the *cap5* regions from strain Newman that are cloned in the complementation plasmids pKOR19 (*cap5ABC* plus truncated *cap5D*), pKOR18 (*cap5ABCD* plus a portion of *cap5E*), pCap16 (*cap5ABCDE*), and pCap17 (which contains *cap5ABCDEF*). (C) CP5 serotyping results from USA300 MRSA strain 923 transformants harboring the *cap5* complementation plasmids depicted in panel B.

were identified previously in three USA300 MRSA isolates from 2004 to 2005 (22).

***cap5* loci in publicly available USA300 *S. aureus* genome sequences.** To confirm that the USA300-specific mutations were not confined to the Chicago and Los Angeles locales, we also analyzed 319 previously published USA300 isolate sequences from San Diego, CA ($n = 35$), and New York ($n = 277$). (Annotations of the included isolates are outlined in Table S2 in the supplemental material.) All but one isolate had all 4 *cap5* mutations identical to those in our USA300 collection.

***cap5* mutations among USA300 *arcA*-negative MRSA and USA300 MSSA isolates.** We performed Sanger sequencing of PCR products amplified from the *cap5* locus to determine if the four *cap5* mutations identified in USA300 MRSA isolates were also conserved among 9 atypical USA300 MRSA isolates that were ACME *arcA* negative and 19 USA300 MSSA isolates (Table 2). All 4 USA300-specific mutations were conserved among 8 of 9 (89%) ACME *arcA*-negative USA300 MRSA isolates tested (Table 2). One USA300 MRSA outlier (isolate 111395F) had only 2 of the 4 *cap* mutations (P_{cap5} −73 and *cap5D*-994). However, this isolate

TABLE 2 Summary of conserved mutations in *cap5* loci detected in USA300 and USA500 isolates in our collection[a]

Gene	nt position	nt in: Strain Newman	nt in: USA300	Codon (aa) in: Strain Newman	Codon (aa) in: USA300	Codon position	nt (no./total) USA300 MRSA ACME *arcA*$^+$ ($n = 155$)[b]	USA300 MRSA ACME *arcA*$^-$ ($n = 9$)[c]	USA300 MSSA ($n = 19$)[c]	USA500 MRSA ($n = 14$)[c]	USA500 MSSA ($n = 2$)[c]
P_{cap5}[d]	−73[e]	T	C	NA[f]	NA	NA	C (155/155)	C (9/9)	C (19/19)	C (14/14)	C (2/2)
cap5D	994[g]	A	AA	AAA (Lys)	STOP	338	AA (155/155)	AA (9/9)	AA (19/19)	AA (14/14)	AA (2/2)
cap5E	223	G	T	GAT (Asp)	TAT (Tyr)	75	T (155/155)	T (8/9) G (1/9)	T (16/19) G (3/19)	G (14/14)	G (2/2)
cap5G	478	T	C	TTC (Phe)	CTC (Leu)	160	C (155/155)	C (8/9) T (1/9)	C (10/19)	C (11/14) T (3/14)	T (2/2)

[a] SNPs of clinical isolates subjected to whole-genome sequencing or Sanger sequencing of PCR products as described in the text.
[b] USA300 ACME *arcA*$^+$ MRSA isolates were characterized by PFGE or by molecular typing as described in Materials and Methods.
[c] Details of the molecular typing characteristics are provided in Table S1 in the supplemental material.
[d] P_{cap5}, promoter region of the *cap5* operon.
[e] Seventy-three nucleotides upstream of the *cap5A* ATG translation initiation codon.
[f] NA, not applicable since the polymorphism is in an intergenic region.
[g] *cap5D* nt 994 in the USA300 reference strain corresponds to *cap5D* nt 1006 in strain Newman.

was obtained in 1995 and is not typical of recent USA300 MRSA isolates. Although isolate 111395F had a SmaI digestion pattern similar to that of a USA300 isolate, it had features consistent with USA500 isolates, including *spa* type t064, ACME *arcA*, and PVL. However, strain 111395F also had features atypical for USA500, including carriage of SCC*mec* II.

Among the USA300 MSSA isolates tested, 16 of 19 (84.2%) had all 4 USA300-specific mutations in P_{cap5}, *cap5D*-994, *cap5E*-223, and *cap5G*-478 (Table 2; see Table S1 in the supplemental material). Three outlier MSSA isolates had just three of the USA300-specific mutations (P_{cap5}, *cap5D*-994, and *cap5G*-478).

USA500 isolates. Among USA500 isolates, the four USA300 *cap5* mutations described above were common but not universal. By Sanger sequencing, we found that 11 of the 14 (78%) USA500 MRSA isolates had 3 of the 4 USA300-specific mutations (P_{cap5} −73, *cap5D-994*, and *capG*-478), and none had the G→T mutation at *cap5E*-223. Three outlier USA500 MRSA isolates and both USA500 MSSA isolates had only 2 of the 4 mutations (P_{cap5} −73 and *cap5D-993*) (Table 2), but these are each sufficient to yield a CP^- phenotype (27).

Analysis of USA300-specific *cap5* mutations in *S. aureus* phylogenies. To evaluate the appearance of the *cap5* mutations on the core phylogeny of *S. aureus*, we analyzed the USA300-specific *cap5* mutations in 319 publicly available USA300 *S. aureus* genome sequences, 146 USA300 MRSA genomes in this study, and 90 non-USA300 genomes from 41 STs (Fig. 4). This allowed us to propose that the USA300 *cap5* mutations evolved in a stepwise fashion. Moreover, these data suggest that USA300 and USA500 appear to have emerged from a common ancestor, and the P_{cap5} T→C mutation likely occurred in the last common ancestor (LCA) of all USA300 and USA500 strains. The *cap5D* insertion may also have occurred in the LCA of both USA300 and USA500 but may have reverted back to wt in a recently emerged branch within USA500 (exemplified by strains GA231 and GA27). The *cap5G* T→C mutation had its origins in an ancestor of all USA300 strains that was also a progenitor of a clade of strains falling between the classical USA300 and USA500 patterns.

Our analysis also suggests that the isolates with ST609 and ST623 are intermediate between USA300 and USA500 and might have a common ancestry. The conserved *cap5* mutations that we describe were not present in any of the other 20 clonal complexes and were not in all ST8 strains. Thus, these mutations are unique to USA300 and its close relatives USA500, ST609, and ST623.

Complementation studies to test the phenotypic effect of *cap5* gene mutations. To determine the phenotypic relevance of the *cap5* single nucleotide polymorphisms (SNPs) in strain USA300, we tested whether expression of wild-type *cap5* genes in *trans* on a complementation plasmid could restore CP5 production to USA300 MRSA strain 923. The *cap5* genes included in each complementation plasmid are depicted in Fig. 3B. Transformation of USA300 strain 923 with pKOR19 (expressing wt *cap5ABC*) or pKOR18 (expressing wt *capABCD*) was not sufficient to restore the $CP5^+$ phenotype (Fig. 3C). This was not due to mutations in the cloned *cap5* promoter or *cap5*D, as confirmed by DNA sequence analysis. In contrast, transformation of USA300 strain 923 with pCap16 (expressing wt *cap5ABCDE*) or pCap17 (wt *cap5ABCDEF* genes) restored CP5 production (Fig. 3C). These data indicate that the mutations in *cap5D* and P_{cap5} in USA300 and USA500 strains ablate CP production. These data also reveal a vital role for the Asp→Tyr substitution at Cap5E-75 in ablating CP production. In contrast, Cap5G tolerates the Phe160Leu substitution in USA300 because complementation of CP5 production in a USA300 strain was achieved by a plasmid lacking *cap5G*.

DISCUSSION

The fact that CP antigens have been used successfully in vaccines against several bacterial pathogens has encouraged a similar vaccine strategy for *S. aureus*. Several vaccines that have been designed for use against *S. aureus* have included CP5 and CP8 because they are the most common CP types produced by *S. aureus* clinical isolates. However, this study shows that USA300 MRSA isolates, as well as USA300 MSSA and closely related USA500 isolates, are universally CP^- negative. We also showed no evidence that USA300 isolates produce CP5 *in vivo* during infection in mice. In contrast, Timofeyeva et al. demonstrated that CP5 was detected *in vivo* on USA300 MRSA after a 6-h infection (28). We cannot explain the discrepancy between the two studies. However, it is unlikely that a strain could revert all 3 mutations that would be required for USA300 to revert to a CP^+ phenotype.

USA300 isolates have increased in prevalence in the United States to become one of the most common *S. aureus* genotypes (15), especially among SSTIs, where they have accounted for 98% of MRSA isolates (16). Because USA300 isolates comprise a substantial portion of the *S. aureus* disease burden in the United States, a vaccine based solely on CP would likely demonstrate low efficacy in a U.S. population.

These results extend the observations reported a decade ago when USA300 MRSA isolates were first reported to be acapsular (22, 23). We now show that the CP^- phenotype has persisted among USA300 MRSA isolates and is a universal and characteristic trait of this highly successful MRSA lineage. Moreover, we have now established that USA300 MSSA isolates and USA500 isolates are also CP^-.

Our prior understanding of the genetic basis of the CP^- phenotype among USA300 isolates was limited to our identification of two mutations in the *cap5* locus in three USA300 isolates (22) that had been shown previously to ablate CP production in other *S. aureus* strains (27, 32). The comprehensive genetic analyses of USA300 isolates in this study reveals that the 4 *cap5* mutations are present in all but 1 USA300 MRSA isolate and in all but 3 USA300 MSSA isolates. Importantly, 3 of those 4 mutations are alone sufficient to ablate CP production. It would therefore require reversion of all 3 mutations in USA300 MRSA to produce a strain that produces a CP. Also, 3 of the 4 same mutations are common in the USA500 lineage. It has been shown previously that the mutations in P_{cap5} −73 and the frameshift mutation in *cap5D* ablate CP production in *S. aureus* (22, 27). Whereas the promoter mutation attenuates expression of *capABCDEF*, the *cap5D* and *cap5E* mutations ablate production or activity of enzymes responsible for producing key soluble precursors UDP-D-FucNAc and UDP-L-FucNAc, respectively, that are needed for the biosynthesis of CP (2, 34). This is the first report to show that Asp75 is a critical residue in Cap5E, which is an essential enzyme for CP5 production (35). This enzyme converts the precursor (UDP-GlcNAc) to a keto-intermediate that is further reduced (by Cap5F) and epimerized at C-2 (by Cap5G) to yield UDP-*N*-acetyl L-fucosamine (36). Thus, USA300 is unable to produce UDP-*N*-acetyl L-fucosamine, which is required for CP5 and CP8 production. When the Cap5E Asp75Tyr conversion is overlaid on the Cap5E three-dimensional crystal struc-

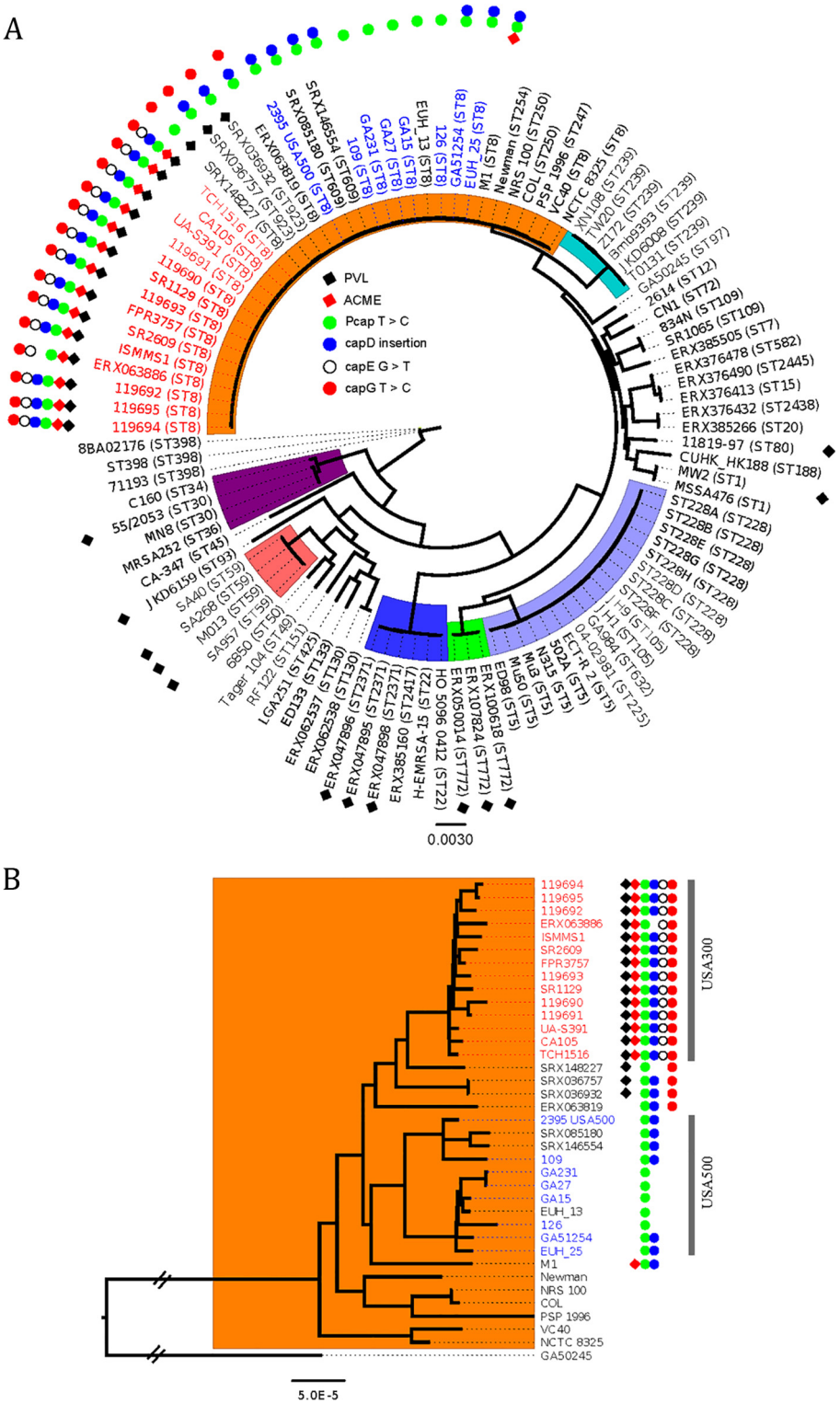


FIG 4 A maximum likelihood phylogeny based on whole-genome alignments of publicly available *S. aureus* genomes. (A) Phylogenetic analysis of 104 *S. aureus* strains from 41 STs showing the distribution of conserved mutations in *cap5* in USA300 (Table 2). There are 14 USA300 strains in the tree that are representative of 146 strains from our study and 319 publicly available USA300 genomes. The remaining 90 genomes are from non-USA300 isolates. Strain names are shown as either the common name or the accession number followed by the sequence type (ST) in parentheses. Strains with red and blue text, respectively, are previously confirmed USA300 and USA500 isolates. The clade in orange is clonal complex 8 (CC8). Other common CCs are also highlighted. For simplicity, only a representative strain from each ST with the same pattern of *cap5* mutations was included. Carriage of PVL and ACME is indicated with black and red diamonds, respectively, due to their association with the USA300 subclone. *cap5* mutations are shown as solid or open circles as indicated. (B) High-resolution phylogeny of CC8 strains (a subset from panel A). USA300 and USA500 strains are annotated as in panel A.

ture (37), it lies within the coenzyme-binding domain. This information could be useful in developing inhibitors of CP biosynthesis.

Our analysis of *S. aureus* whole-genome sequences (WGSs) in the public databases from 41 STs also provides insight into the evolution of the USA500 and USA300 *S. aureus* lineages not previously appreciated. All USA500 isolates that we evaluated had at least 2 of the 4 *cap5* mutations found in USA300 MRSA (in P_{cap5} and in *cap5D*—both essential for CP production [36]). Moreover, most USA500 MRSA isolates had the *cap5G*−478 T→C mutation. Notably, because none of the USA500 isolates had the *cap5E*-223 G→T mutation, this is a feature in the core genome that might be used to distinguish USA500 from USA300 isolates in future epidemiological studies. By overlaying the USA300 mutations on the phylogeny of *S. aureus* genomes (Fig. 4), it is apparent that the 4 USA300 *cap5*-specific mutations only exist in clonal cluster 8, and they arose sequentially, starting with P_{cap5}, in a common ancestor of USA300 and USA500 isolates. Importantly, this is the first study to reveal that USA300 and USA500 lineages inherited the *cap* mutations from a common ancestor rather than USA300 inheriting them from USA500, as suggested previously (21).

MRSA isolates arise by horizontal acquisition of a mobile genetic element called SCC*mec* that carries the methicillin resistance gene, *mec* (18). Interestingly, only a limited number of genetic backgrounds have become successful MRSA clones (18). Despite the close genetic similarity between the USA300 and USA500 lineages (21, 26, 30), USA300 has disseminated more widely in the United States. Comparisons of virulence factors produced by USA500 versus USA300 may provide insight into their relative successes. USA500 and USA300 MRSA isolates both exhibit high-level virulence in animal infection (22, 26, 30), and both express high levels of global regulators and exotoxins encoded in the core genome (22, 26). CP is another core virulence determinant that we have ruled out as a key player in the relative success of USA300 over USA500, because CP is not expressed in either subclone. The success of USA300 may lie in the fact that it carries ACME, which is thought to enhance transmissibility and competitiveness of USA300, although not its virulence (19, 20, 22).

Our findings shed light on the roles of CP5 and CP8 in staphylococcal virulence of USA300 and USA500 strains. CP has been shown to protect *S. aureus* from opsonophagocytic killing by human neutrophils (11, 29) and thus protects *S. aureus* from host immune killing. Moreover, the capsule has been shown to enhance virulence in animal models of bacteremia, subcutaneous and renal abscess formation, surgical wound infection, septic arthritis, and lethality (2). Yet its absence in USA300 and USA500 strains underscores the fact that a CP is not necessary for virulence of these strains. This is consistent with studies that have shown that CP production can attenuate staphylococcal virulence in situations in which bacterial adherence is critical, as in endocarditis and murine mastitis, because it masks the adhesins on the cell surface (2). USA300 is best known for causing SSTIs, so we can speculate that perhaps this niche favors a CP^- phenotype by exposing adhesins on the surface of the bacteria. Because the pathogenesis of staphylococcal infections is multifactorial, it is likely that other virulence factors produced by USA300 and USA500 compensate for lack of CP during infection. Overexpression of *agr*, *sae*, α-hemolysin, and a variety of leukocidins (22), may even have been selected for in the absence of a CP.

Our study has limitations. Our sample of isolates was obtained mainly from patients in Chicago and Los Angeles. This was offset by analyzing public genome sequences, which supported and extended the findings from our isolates.

We conclude that a vaccine designed for a U.S. population should not be solely based on CP antigens because USA300 and USA500 CP^- isolates cause a large proportion of *S. aureus* infections.

MATERIALS AND METHODS

Determination of USA300 and USA500 genetic backgrounds. *S. aureus* isolates were confirmed with a catalase test and by agglutination using Staphaurex Plus (Remel) and underwent genotyping by multilocus sequence typing (MLST) (38), Ridom *spa* typing (39), SCC*mec* typing (40), and PCR detection of *mecA*, the ACME-borne *arcA* gene (ACME *arcA*) (19), and genes encoding PVL (41) as described previously (39). USA300 MRSA isolates were classified by SmaI digestion patterns using pulsed-field gel electrophoresis (PFGE) as described previously (39). In the absence of PFGE data, the USA300 genotype was inferred in MRSA isolates that belonged to ST8 and carried SCC*mec* type IV and PVL genes (39). All USA500 isolates and USA300 MSSA isolates were identified solely by PFGE using SmaI digestion patterns. PFGE patterns were assigned to a given clonal group by comparison to a reference strain using an 80% similarity cutoff using the Dice coefficient in BioNumerics software (Applied Maths, TX) (42). Isolates were obtained from consenting patients as approved by the Institutional Review Board of the participating institutions.

CP5 and CP8 serotyping. Capsule serotyping was performed by a colony immunoblot method with the use of CP5- and CP8-specific polyclonal antibodies as described (43). Briefly, tryptic soy agar plates were spot inoculated in a grid pattern with up to 60 *S. aureus* isolates and incubated overnight at 37°C. The colonies were blotted onto nitrocellulose filter membranes (diameter, 82.5 mm) for 5 min at ambient temperature. Adherent colonies were fixed to the membranes by being heated at 60°C for 15 min. After being washed twice in phosphate-buffered saline (PBS) to remove excess cells, the filters were immersed in a solution of trypsin (1 mg/ml) for 60 min at 37°C to remove protein A. After two washes in PBS, the filters were blocked with 0.05% skim milk for 1 h and washed in PBS containing 0.05% Tween 20 (PBST). Capsule type-specific polyclonal antiserum (diluted 1:1,000) was incubated with each filter at 37°C for 1 h. After being washed in PBST, horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin was incubated with each filter for 1 h at 37°C. After three washes in PBST and two washes in PBS, substrate (3 mg 4-chloro-1-naphthol per ml of methanol diluted 1:5 in PBS and containing 0.1% H_2O_2) was added to the filters. A purple color developed within 15 min and was scored visually from 0 to 4+. Positive reactions were scored as 2+ to 4+. The reactivity of the isolates was evaluated by comparison to those of control *S. aureus* strains ($CP5^+$, $CP8^+$, and CP^- isolates) included on each filter membrane. Isolates with no reaction to CP-specific antibodies were defined as CP^-.

USA300 strains subjected to whole-genome sequencing. To determine the basis for the $CP5^-$ phenotype, we analyzed the entire *cap5* locus from the WGS assemblies we produced (33) from 146 USA300 MRSA isolates that were included in the sample tested for CP production. These isolates were obtained between 2008 to 2010 from the University of Chicago Medical Center ($n = 75$) and Harbor-UCLA Medical Center in Los Angeles ($n = 71$) (44).

WGS and SNP calling. Genomic DNA was sequenced on an Illumina HiSeq 2000 (Illumina Inc., San Diego, CA) at ~215× the median depth of coverage per strain as described in reference 33 and the supplemental material. Mutations in the *cap5* locus were retrieved from the assemblies by comparison to the *cap5* locus of the $CP5^+$ reference strain Newman using Jalview (45). All nucleotide positions are reported relative to the USA300 reference strain TCH1516.

Confirmation of *cap5* mutations. To evaluate whether the conserved mutations we identified in the WGS analysis were present in *S. aureus* isolates of interest, the regions surrounding the mutations were PCR am-

TABLE 3 Sequences of the primers used for PCR amplification and sequencing of the *cap5* gene fragments

Primer (expected size [bp])[a]	Sequence
cap5A promoter, P_{cap5}	
Forward	5′ GAATCATTAGCTAAAGCTGTCTAC 3′
Reverse	5′ GTCACCCTTAGTTTGATTCA 3′
cap5D (803)	
Forward	5′ GTAAAATTGCGGATATTCCAGAAC 3′
Reverse	5′ AGTGGAATCACAGATCCTCT 3′
cap5E (381)	
Forward	5′ GCACAGGATCATTCGGTAAT 3′
Reverse	5′ CTTTTGAAATACCCATAGCA 3′
cap5G (543)	
Forward	5′ TGGAAGCGGGTAATAGATGC 3′
Reverse	5′ GGACACCAGGGAACTTCAAA 3′

[a] Forward primers have a sense orientation, and reverse primers have an antisense orientation.

plified from the indicated strains using primers listed in Table 3. The amplification products were subjected to Sanger sequencing in the DNA sequencing and genotyping facility (University of Chicago) using the amplification primers. Mutations were identified using Vector NTI software (Invitrogen) by alignment to the *cap5* locus of the $CP5^+$ reference strain Newman.

***cap5* mutations in publicly available USA300 and non-USA300 *S. aureus* genomes.** To examine whether the four *cap5* locus mutations found in the WGSs of our sample of 146 USA300 MRSA isolates were universal in this clonal lineage, we also interrogated *cap5* gene cluster sequences from 319 publicly available USA300 MRSA genomes. These included 35 isolates from San Diego (46), 277 isolates from New York City (47), the completed genome sequences of strains TCH1516 (48), FPR3757 (49), UA-S391 (50), and ISMMS1 (51), and three isolates from Alam et al. (52). Also, the phylogenetic distribution and origin of the *cap* mutations among 90 publicly available non-USA300 *S. aureus* genomes from 41 STs were studied using whole-genome alignment-based maximum likelihood phylogeny of publicly constructed genomes using PhyML, as implemented in REALPHY (53). Further details of the public sequence annotations are provided in Table S2 in the supplemental material.

Complementation studies of the *cap* locus. In order to test the effect of the *cap5* mutations, we electrotransformed (54) a USA300 MRSA strain, 923, with plasmids containing various *cap5* genes that were isolated from strain Newman and cloned into the vector pCU1 as summarized in Table 4. Briefly we used pKOR18 (which contains *cap5ABCD* and a portion of *cap5E*), pKOR19 (which contains *cap5ABC* and truncated *cap5D*) (27), pCap16 (which contains intact *cap5A-E*) (35), and pCap17 (which contains intact *cap5A-F*) (35). Following transformation, strain 923 was cultured in the presence of chloramphenicol at 5 mg/liter to maintain the plasmid. CP5 serotyping was performed as described above.

Nucleotide sequence accession number. The raw sequence reads from the project have been deposited into the National Center for Biotechnology Information (NCBI) Short Read Archive (SRA) database under accession no. SRP039020. These sequences were also analyzed in reference 33.

TABLE 4 Plasmid constructs used in *cap5* genetic complementation experiments

Plasmid[a]	*cap5* genes contained in plasmid	Reference
pKOR19	P_{cap5} + *capABC*	27
pKOR18	P_{cap5} + *capABCD*	27
pCap16	P_{cap5} + *capABCDE*	35
pCap17	P_{cap5} + *cap5ABCDEF*	35

[a] pCap16 and pCap17 were kindly provided by Timothy Foster.

SUPPLEMENTAL MATERIAL:

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02585-14/-/DCSupplemental.

Text S1, DOCX file, 0.1 MB.
Figure S1, TIF file, 0.5 MB.
Table S1, PDF file, 0.1 MB.
Table S2, PDF file, 0.1 MB.

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